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Taxonomic Reassessment of Strains formerly Classified as *Chloromonas insignis* (Volvocales, Chlorophyceae) and Description of *Gloeomonas anomalipyrenoides* sp. nov.

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Two strains previously identified as *Chloromonas insignis*, NIES-447 and ACOI-3241, and two new strains related to NIES-447 were examined using light microscopy. The molecular phylogeny of their 18S *r*RNA gene sequences were also examined, and their identifications are reassessed. The strains represent two independent species, both of which can be distinguished from *Chloromonas insignis*. Strain NIES-447 and the two new strains each have large, irregular atypical pyrenoids without associated starch granules. They are described here as *Gloeomonas anomalipyrenoides* Nakada *et al*. sp. nov. Strain ACOI-3241, which has small atypical pyrenoids on the tips of the chloroplast lobes, was identified as *C. vernalis* (Skuja) Nakada comb. nov. The identity of the three strains of *G. anomalipyrenoides* was verified by their identical ITS-2 *r*DNA sequences. Both *G. anomalipyrenoides* and *C. vernalis* of the clade *Chloromonadinia* in the Volvocales (Chlorophyceae) are closely related to other species of *Gloeomonas* and to *C. kasaiae* Matsuzaki, Nakada, Y. Hara & Nozaki, respectively.

Keywords: 18S *r*RNA, *Chloromonas*, culture strain, *Gloeomonas*, light microscopy, new species, phycology, phylogeny

Chloromonas Gobi and *Gloeomonas* G.A. Klebs (Volvocales, Chlorophyceae) are unicellular green biflagellates traditionally characterized by unflattened cells lacking a distinct pyrenoid in the chloroplast (e.g. Ettl 1983, Melkonian & Preisig 2000). These two genera are distinguished from each other primarily by the distance between the bases of their two flagella (close in *Chloromonas* and distant in *Gloeomonas*). In the redefined genus *Chloromonas*, primarily based on a phylogenetic tree, Pröschold *et al*. (2001) included both pyrenoid bearing and pyrenoid lacking species in *Chloromonadinia*. Nozaki *et al.* (2010) showed that the same clade includes several species of *Gloeomonas*. *Chloromonas* is now treated as containing species of *Chloromonadinia* but excluding traditional *Gloeomonas* (note that *Gloeomonas* G.A. Klebs 1888 has priority over *Chloromonas* Gobi 1899/1900). Because *Chloromonas* in this sense is paraphyletic, division of *Chloromonas* into monophyletic genera is necessary for phylogenetic classification. *Chloromonas* should be redefined as a clade including the type species *Chloromonas reticulata* (Gorozh.) Gobi, but excluding the type species of *Gloeomonas* (*Gloeomonas ovalis* G.A. Klebs), whose phylogenetic position is yet to be determined.

Also, slight separation of the flagellar bases is known in some species of *Chloromonas* (e.g. *Chloromonas rubrifilum* (Korshikov) Pröschold *et al.* SAG 3.85 and the formerly recognized *C. insignis* NIES-447). Therefore, the intermediate forms of *Gloeomonas* should be closely examined before *Chloromonas* is recharacterized and reclassified.

The strain previously identified as *Chloromonas insignis* NIES-447 is the sister strain of the genus *Gloeomonas* (Nozaki *et al.* 2010), but detailed light microscopy of the strain had not been reported. Nozaki *et al.* (2010) reported moderate separation of the flagellar bases of strain NIES-447, while Anachin (1931) showed the bases of the flagellae of *C. insignis* (Anakhin) Gerloff & H. Ettl to be attached. We therefore examined the morphology of strain NIES-447 and compared it with similar strains, including the putative *C. insignis* ACOI-3241 and two new strains of *Chloromonas/Gloeomonas.* We also examined the phylogenetic position of each of these strains based on the 18S *r*RNA gene. We also examined the ITS-2 sequences of NIES-447 and similar strains.

Materials and Methods

Strain isolation and cultivation

Isolations and re-isolations were performed according to the pipette-washing method (Pringsheim 1946, Andersen & Kawachi 2005). Chlamydomonad strain 97-0808-G-2 was collected from the Grienericksee near Rheinsberg Palace, Rheinsberg, Germany (53°6′ N, 12°53′ E), on 7 August 1997 and was isolated on 8 August. Two sublineages, a wild-type sublineage (97-0808-G-2-WT) and a stigma-less mutant (97-0808-G-2-stg), were derived from strain 97-0808-G-2. Another strain, HkCl-5-3, was collected from Lake Hakkaku, an artificial lake in Togane, Togane-shi, Chiba, Japan (35°33′42″ N, 140°21′34″ E), on 18 June 2005 and was isolated on 19 June. The collections identified as NIES-3641 (=97-0808-G-2-WT), NIES-3642 (=97-0808-G-2-stg) and NIES-3640 (=HkCl-5-3) were deposited in the Microbial Culture Collection at the National Institute for Environmental Studies (NIES; http://mcc.nies.go.jp/top.jsp). One strain identified as *Chloromonas insignis* NIES-447 was obtained from NIES. Strains identified as *C. insignis* ACOI-3241 and *Gloeomonas tubulosa* (Pascher & Jahoda) H. Ettl ACOI-1541 were obtained from the Coimbra Collection of Algae (ACOI; http://acoi.ci.uc.pt/). Because the cultures of ACOI-1541 and 3241 were originally contaminated by bacteria and fungi, the algal cells were re-isolated by the pipette-washing method. The strains were maintained in screw-cap tubes (18 × 150 mm) containing 9–10 mL of C medium (Ichimura 1971) supplemented with vitamins (1 μg/L vitamin B_6, 1 μg/L vitamin B_{12}, and 2 μg/L biotin), at 20°C under a light:dark cycle of 14:10 provided by cool white fluorescent lamps at about 30–100 $\mu mol/m^{-2}/s^{-1}$.

Observations

We observed the 7-d-old cultures grown in C medium by light microscopy using a Leica DM2500 microscope equipped with Nomarski interference optics (Leica, Wetzlar, Germany) and an Olympus DP71 digital camera (Olympus, Tokyo, Japan).

DNA sequencing

The 18S *r*RNA gene sequences of strains 970808-G-2-WT, 970808-G-2-stg, HkCl-5-3, ACOI-1541, and ACOI-3241 (AB971357–AB971361), and the ITS-2 sequences of NIES-447, 97-0808-G-2-WT, 97-0808-G-2-stg, and HkCl-5-3 (AB971362–AB971365) were determined. DNA extraction, polymerase chain reaction (PCR), and sequencing were performed as described previously (Nakada *et al.* 2010a). The previously published primers 18S-FA, RB, FC, RD, FE, RF, FA2, and RB2 (Nakazawa & Nozaki 2004, Nakada *et al.* 2007, 2010b) were used to amplify 18S *r*RNA, and the previously published primers ITS-a, ITS-b, and ITS-c (Coleman *et al.* 1994) were used to amplify ITS-2.

Phylogenetic analyses.

Based on preliminary analyses, all of the 18S *r*RNA gene sequences examined were affiliated with the clade *Chloromonadinia* (Nakada *et al.* 2008, Matsuzaki *et al.* 2013), and unambiguously aligned with other sequences of *Chloromonadinia*. Sequences of *Actinochloris* sp. BCP-LG3VF20 (AY271674) and an uncultured chlorophyte WIM12 (AM114821) were used outgroups (Nakada *et al.* 2008). The alignment used for the phylogenetic analyses corresponded to positions 49–1677 of *Volvox carteri* F. Stein f. *nagariensis* M.O.P. Iyengar (Rausch *et al.* 1989; X53904).

Bayesian phylogenetic analyses were performed using MrBayes 3.2.1 (Ronquist *et al.* 2012) with 3,000,000 generations of Markov chain Monte Carlo iterations, discarding the first 25% as burn-in. Bootstrap analyses (Felsenstein 1985) of maximum likelihood were performed with 100 replications and nearest-neighbor interchange heuristic searches started with a neighbor-joining tree. Bootstrap analyses of maximum parsimony with 1000 replications, and those of neighbor-joining method (Saitou & Nei 1987) with 1000 replications using Jukes and Cantor distances, were performed using PAUP* 4.0b10 (Swofford 2002) as described by Nakada & Nozaki (2009). The evolutionary models for Bayesian inference (GTR + I + G) and maximum likelihood analysis (GTR + I + G) were selected using MrModeltest 2.2 (Nylander 2004) and Modeltest 3.7 (Posada & Crandall 1998), respectively.

Preparation of resin embedded specimen

Cells from 7-d cultures of NIES-447 and ACOI-3241 in AF-6 medium (Kato 1982, modified as in Andersen *et al.* 2005) were fixed with 1% OsO_4 and 2.5% glutaraldehyde for 1 h on ice. The fixed materials were dehydrated in an ethanol series and then substituted with propylene oxide and finally Spurr's resin (Spurr 1969). The resin-embedded materials were mounted on glass slides with cover slips for microscopy.

Results and Discussion

Strains NIES-447, 97-0808-G-2, and HkCl-5-3 have mutually distant flagellar bases, while strain ACOI-3241 had close flagellar bases (Figs. 1, 2). All the strains belong to the clade *Chloromonadinia* (Fig. 3). We determined strains NIES-447, 97-0808-G-2 and HkCl-5-3, based on the traditional definition, to be a species of *Gloeomonas*. We identified strain ACOI-3241 as a species of *Chloromonas* (here, *Chloromonas* is a paraphyletic group containing species of clade *Chloromonadinia* but excluding traditional *Gloeomonas*).

Based on our observations using light microscopy (Figs. 1, 2; see *Remarks.* for each species), strains NIES-447, 97-0808-G-2 and HkCl-5-3 were determined to be a new species of *Gloeomonas*, here described as *G. anomalipyrenoides* Nakada *et al.* sp. nov. Strain ACOI-3241 was reclassified as *C. vernalis* (Skuja) Nakada comb. nov.

Gloeomonas anomalipyrenoides Nakada, Matsuzaki & Nozaki **sp. nov.** — Fig. 1.

Typus. Specimen TNS-AL-58902 (**holotype**; Fig. 4) conserved at TNS (National Museum of Nature and Science, Tsukuba, Japan); permanent preparations of vegetative cells were derived from culture strain NIES-447.

Description. (Based on NIES-447, 97-0808-G-2-WT, and HkCl-5-3) Mature vegetative cells broadly ellipsoid to nearly spherical (Fig. 1A–G). Cell wall distinct, with broad keel-like papilla (Fig. 1B, C, E, G, H, J, K). Papilla low and dome-shaped in front view (Fig. 1J), widely triangular in side view (Fig. 1K). Cells usually covered by mucus layer 1–1.5 μm in thickness (Fig. 1H). Flagella two, emerging from edges of papilla, base covered by inconspicuously short sheaths extending from cell wall (Fig. 1J). Flagella 1.0–1.5(–1.7) × length of cell. Basal bodies separate from each other (Fig. 1J). Chloroplast deep-cup shaped, surface smooth or weakly reticular (Fig. 1A, D, F). Pyrenoids without associated starch, but with few long irregularly branched and sometimes reticulate regions corresponding to atypical pyrenoids on inner surface of chloroplast (Fig. 1B, C, E, G). Stigma generally present, circular to short elliptical, in middle to anterior region of cell (Fig. 1A, F), or sometimes two, one in anterior to middle region, one in middle to posterior region (Fig.

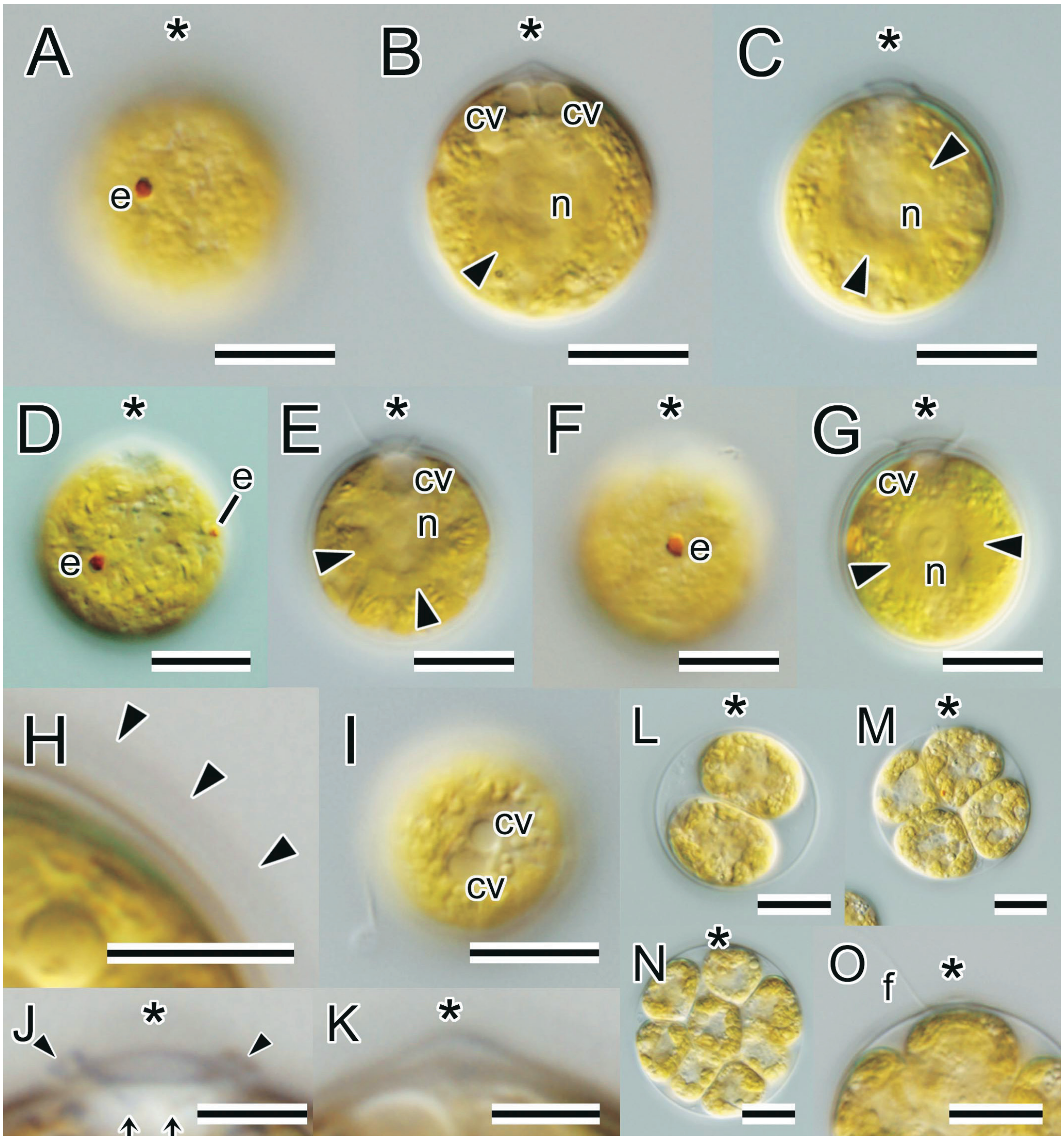

FIG. 1. *Gloeomonas anomalipyrenoides* Nakada *et al.* sp. nov. (A–C, H, K–O: NIES-447, D: 97-0808-G-2-WT=NIES-3641, E, I, J: 97-0808-G-2-stg=NIES- 3642, F, G: HkCl-5-3=NIES- 3640). Cells in 7-d-old cultures. Asterisks indicate the anterior direction of the cells or zoosporangia. A, D, F: Surface views of vegetative cells (D: Two stigmata are shown, one of normal size, and the other small). B, C, E, G: Optical sections of vegetative cells (B: Side view. C, E, G: Front view). Pyrenoid regions within the chloroplast are indicated by arrowheads. H: Mucus coverage (indicated by arrowheads) outside the cell wall. I: Cells from top view, showing two contractile vacuoles. J: Papilla from front view. Flagellar sheaths and proximal ends of basal bodies are indicated by arrowheads and arrows, respectively. Note that flagella were lost during preparation. K: Papilla from side view. L–O: Zoosporangia including two (L), four (M), and eight (N, O) daughter cells. O: Zoosporangia with a remaining flagellum from a mother cell. cv, contractile vacuole; e, stigma; f, flagellum; n, nucleus. Scale bars = 10 µm (A–G, I, L–O), 5 µm (H), or 3 µm (J, K).

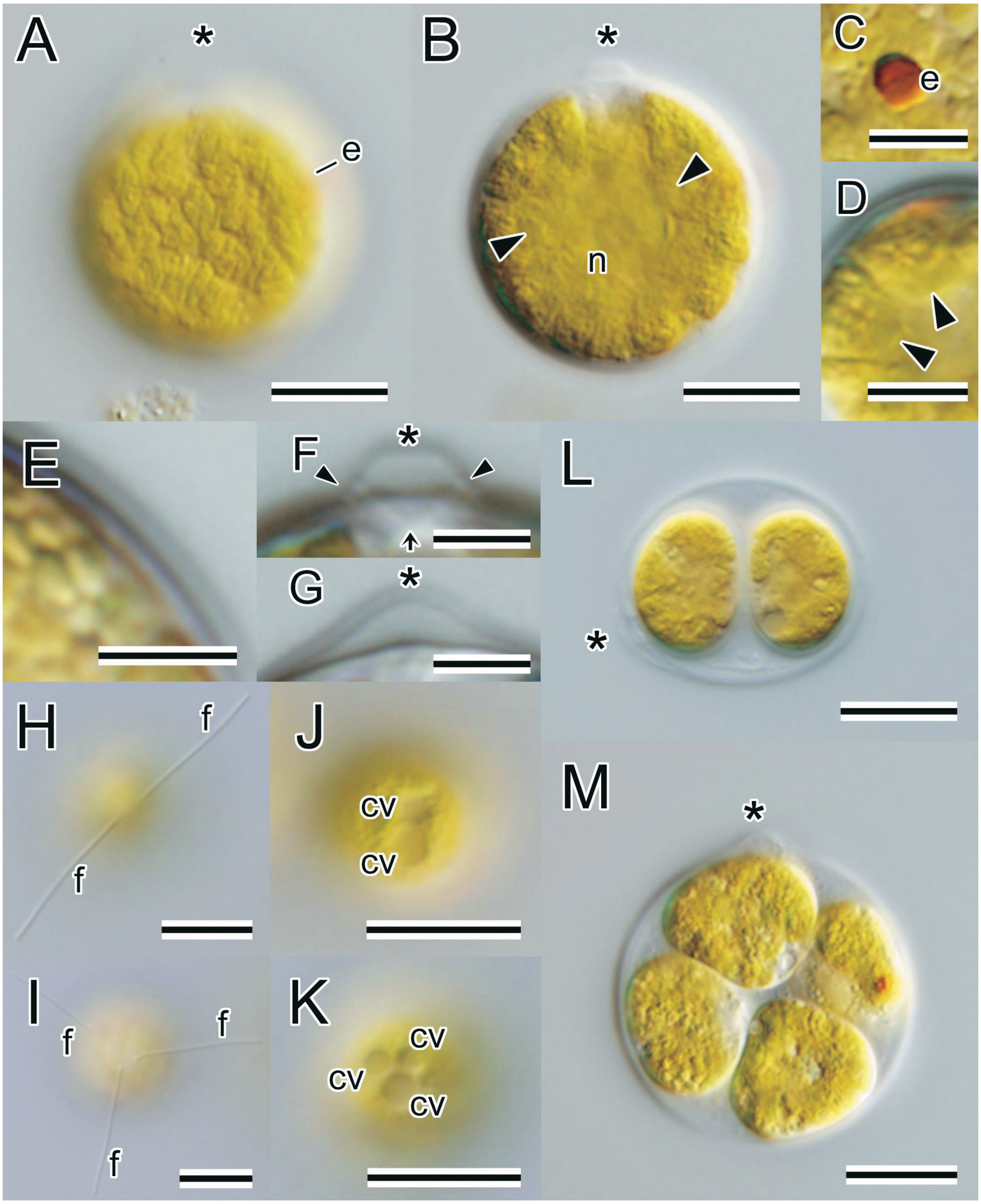

FIG. 2. *Chloromonas vernalis* (Skuja) Nakada comb. nov. (ACOI-3241). Cells in 7-d-old cultures. Asterisks indicate the anterior direction of the cells or zoosporangia. A: Surface view of a vegetative cell. Position of the stigma is indicated (out of focus). B: Optical section of the same cell. Pyrenoid regions are indicated by arrowheads. C: Stigma. D: Pyrenoid regions (indicated by arrowheads) in chloroplast. E: Bilayer cell wall. F: Papilla from front view. Flagellar passages and proximal ends of basal bodies are indicated by arrowheads and an arrow, respectively. Note that flagella were lost during preparation. G: Papilla from side view. H–K: Cells from top views. H: Typical cell with two flagella. I: Cell with three flagella. J: Typical cell with two contractile vacuoles. K: Cell with three contractile vacuoles. L, M: Zoosporangia including two (L) and four (M) daughter cells. For abbreviations, see the legend to Fig. 1. Scale bars = 10 μm (A–B, H–M), 5 μm (C, D), or 3 μm (E–G).

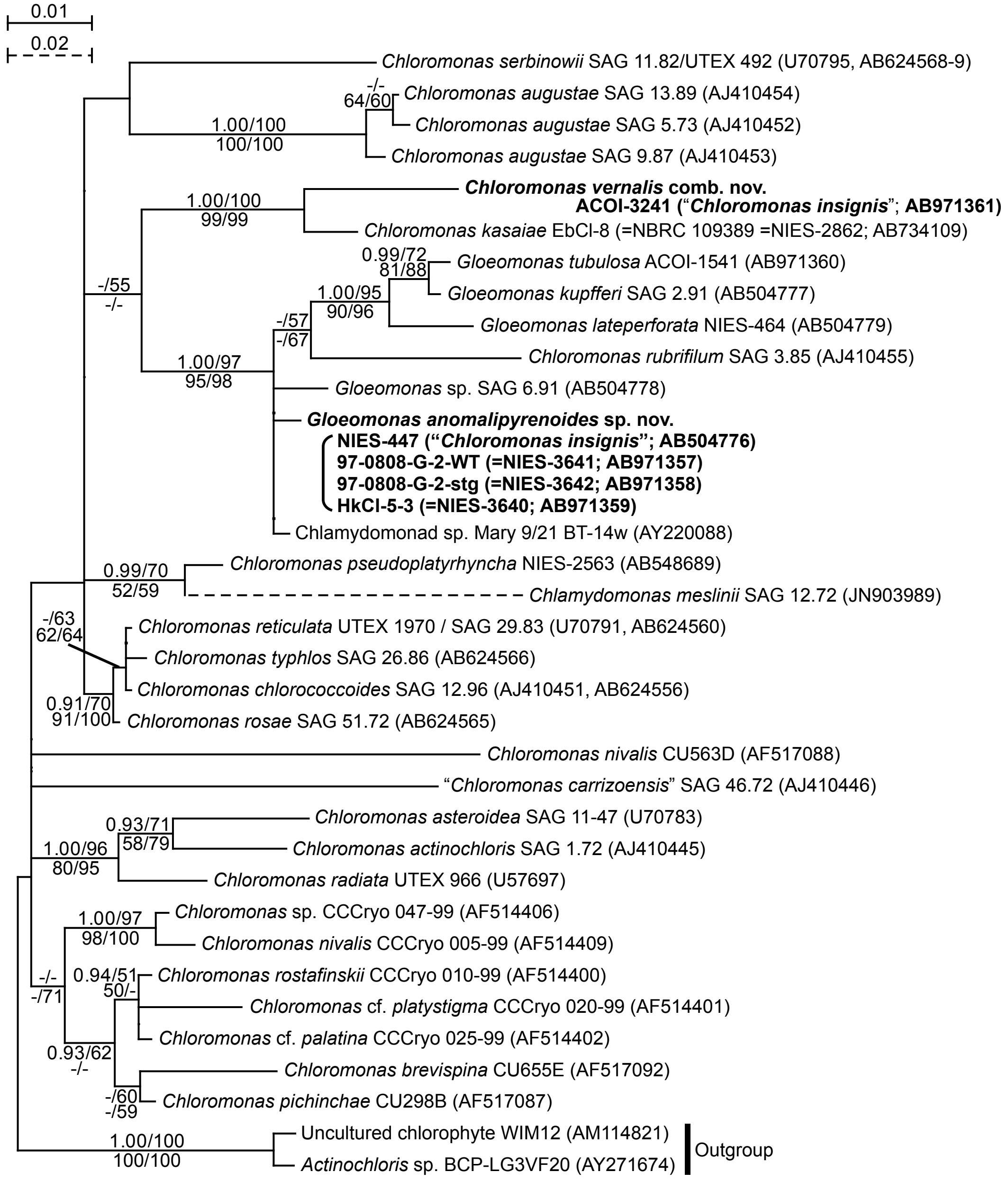
0.01
0.02
Chloromonas serbinowii SAG 11.82/UTEX 492 (U70795, AB624568-9)
-/-
64/60
Chloromonas augustae SAG 13.89 (AJ410454)
1.00/100
100/100
Chloromonas augustae SAG 5.73 (AJ410452)
Chloromonas augustae SAG 9.87 (AJ410453)
1.00/100
99/99
Chloromonas vernalis comb. nov.
ACOI-3241 ("Chloromonas insignis"; AB971361)
Chloromonas kasaiae EbCl-8 (=NBRC 109389 =NIES-2862; AB734109)
0.99/72
81/88
Gloeomonas tubulosa ACOI-1541 (AB971360)
-/55
-/-
1.00/95
90/96
Gloeomonas kupfferi SAG 2.91 (AB504777)
-/57
-/67
Gloeomonas lateperforata NIES-464 (AB504779)
Chloromonas rubrifilum SAG 3.85 (AJ410455)
1.00/97
95/98
Gloeomonas sp. SAG 6.91 (AB504778)
Gloeomonas anomalipyrenoides sp. nov.
NIES-447 ("Chloromonas insignis"; AB504776)
97-0808-G-2-WT (=NIES-3641; AB971357)
97-0808-G-2-stg (=NIES-3642; AB971358)
HkCl-5-3 (=NIES-3640; AB971359)
Chlamydomonad sp. Mary 9/21 BT-14w (AY220088)
0.99/70
52/59
Chloromonas pseudoplatyrhyncha NIES-2563 (AB548689)
Chlamydomonas meslinii SAG 12.72 (JN903989)
-/63
62/64
Chloromonas reticulata UTEX 1970 / SAG 29.83 (U70791, AB624560)
Chloromonas typhlos SAG 26.86 (AB624566)
Chloromonas chlorococcoides SAG 12.96 (AJ410451, AB624556)
0.91/70
91/100
Chloromonas rosae SAG 51.72 (AB624565)
Chloromonas nivalis CU563D (AF517088)
"Chloromonas carrizoensis" SAG 46.72 (AJ410446)
0.93/71
58/79
Chloromonas asteroidea SAG 11-47 (U70783)
1.00/96
80/95
Chloromonas actinochloris SAG 1.72 (AJ410445)
Chloromonas radiata UTEX 966 (U57697)
1.00/97
98/100
Chloromonas sp. CCCryo 047-99 (AF514406)
Chloromonas nivalis CCCryo 005-99 (AF514409)
-/-
-/71
0.94/51
50/-
Chloromonas rostafinskii CCCryo 010-99 (AF514400)
Chloromonas cf. platystigma CCCryo 020-99 (AF514401)
Chloromonas cf. palatina CCCryo 025-99 (AF514402)
0.93/62
-/-
Chloromonas brevispina CU655E (AF517092)
-/60
-/59
Chloromonas pichinchae CU298B (AF517087)
1.00/100
100/100
Uncultured chlorophyte WIM12 (AM114821)
Actinochloris sp. BCP-LG3VF20 (AY271674)
Outgroup

FIG. 3. Bayesian phylogenetic tree of the clade *Chloromonadinia* (Volvocales) based on 18S *r*RNA gene sequences, showing the phylogenetic positions of *Gloeomonas anomalipyrenoides* and *Chloromonas vernalis* (shown in bold). Sequences of *Actinochloris* sp. BCP-LG3VF20 (AY271674) and the uncultured chlorophyte WIM12 (AM114821) were used as outgroups. The corresponding posterior probabilities (≥0.90; top left) and the bootstrap proportions (≥50%) from maximum likelihood (top right), maximum parsimony (bottom left), and neighbor-joining (bottom right) analyses are shown next to the branches. Branch lengths and the scale bar represent the expected number of nucleotide substitutions per site. Accession numbers from the International Nucleotide Sequence Database Collaboration (INSDC; http:// http://www.insdc.org) for each gene are indicated in parentheses.

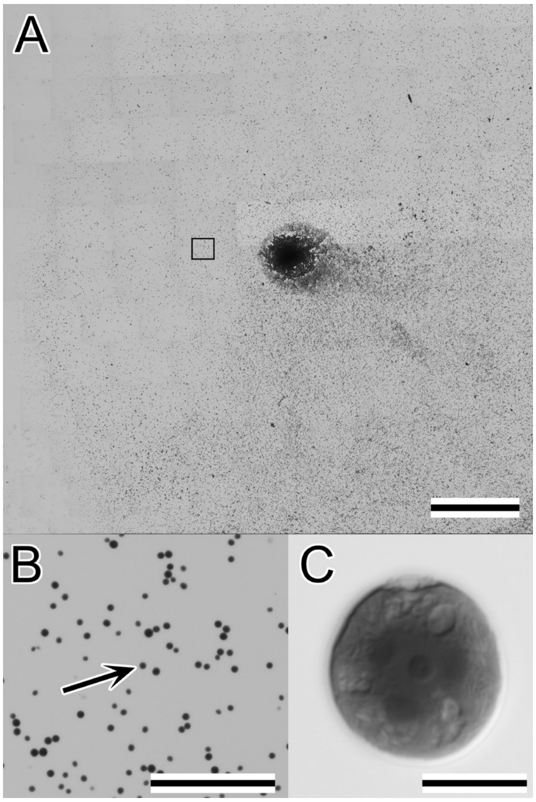

FIG. 4. Permanent preparation of *Gloeomonas anomalipyrenoides* Nakada *et al*. sp. nov. (TNS-AL-58902; holotype). A: Overview of the preparation, shown as a composite micrograph. The region enclosed in a square is enlarged in (B). B: Vegetative cells in the holotype. Arrow indicates the cell shown in (C). C: Detail of a fixed vegetative cell in the holotype. Scale bars = 3 mm (A), 300 µm (B), or 10 µm (C).

1D). Nucleus in center of cell (Fig. 1B, C, E, G). Contractile vacuoles two, close together in apical region (Fig. 1B, I). Cells 11–25 µm in diameter. Asexual reproduction by formation of two, four or eight zoospores (Fig. 1L–N). Flagella sometimes active throughout asexual reproduction (Fig. 1O).

Phylogeny. The 18S *r*RNA gene sequences of the three strains of *Gloeomonas anomalipyrenoides* (NIES-447, 97-0808-G-2-WT, 97-0808-stg, and HkCl-5-3) were identical. During Bayesian analysis (Fig. 3), *G. anomalipyrenoides* formed a clade with other species and strains of *Gloeomonas*, including *G. kupfferi* (Skuja) Gerloff SAG 2.91, *G. lateperforata* (Skuja) H. Ettl NIES-464, *G. tubulosa* ACOI-1541 and *Gloeomonas* sp. SAG 6.91, *Chloromonas rubrifilum* SAG 3.85, and with chlamydomonad Mary 9/21 BT-14w [1.00 posterior probability (PP) and 95–98% bootstrap proportions (BPs)]. However, the sister group of *G. anomalipyrenoides* could not be resolved. Previous three gene (18S *r*RNA, *atp*B, and *psa*B) phylogenies showed that *G. anomalipyrenoides* is sister to the other species of *Gloeomonas*, excluding *C. rubrifilum* (Nozaki *et al*. 2010, Matsuzaki *et al*. 2013; note that *G. tubulosa* was not included in the analyses).

ITS-2 sequences. The ITS-2 sequences of strains NIES-447, 97-0808-G-2-WT, 97-0808-G-2-stg, and HkCl-5-3 were identical (AB971362–AB971365).

Distribution. Germany (97-0808-G-2) and Japan (NIES-447 and HkCl-5-3).

Strains. NIES-447 (authentic strain; labeled *Chloromonas insignis*), 97-0808-G-2 (97-0808-G-2-WT=NIES-3641, 97-0808-G-2-stg=NIES-3642), and HkCl-5-3 (=NIES-3640).

Etymology. The epithet *anomalipyrenoides* refers to the irregular and atypical pyrenoid characteristic of the species [*anomalus* (Latin, adjective), abnormal; *pyrenoides* (Latin, feminine noun), a pyrenoid].

Remarks. The ITS-2 sequences of the strains from Japan and Germany were identical, thereby supporting the species identity of these strains. Their morphology was nearly identical, except that mutant 97-0808-G-2-stg lacked a stigma (Fig. 1A–G). The loss of a stigma was previously reported in a strain of *Chlorogonium euchlorum* (Ehrenb.) Ehrenb. (Nozaki *et al*. 1998), so the presence or absence of a stigma is not a consistent and specific diagnostic characteristic for volvocalean flagellates.

Observations from transmission electron microscopy of *Gloeomonas anomalipyrenoides* NIES-447 was previously reported by Morita *et al*. (1999). In the micrographs, the irregular and atypical pyrenoid regions without starch granules and with pyrenoid regions penetrated by flattened thylakoids are apparent.

The flagellar bases in strains NIES-447, 97-0808-G-2, and HkCl-5-3 are clearly separated (Fig. 1J). The strains are therefore identifiable as species of *Gloeomonas*, rather than *Chloromonas*. Our phylogenetic analyses also support the assignment of these strains to *Gloeomonas* (Fig. 3). Although NIES-447 was originally identified as *C. insignis*, it is distinguished from *C. insignis* by the smooth surface of its chloroplast (Fig. 1A, D, F), separate flagellar bases (Fig. 1J) and broader papilla (Fig. 1J).

Among the species of *Gloeomonas*, *G. anomalipyrenoides* is most similar to *G. mucosa* in having chloroplasts with smooth surfaces in the spherical vegetative cells. *Gloeomonas mucosa*, however, is covered by a thick mucus layer (>5 µm, judged from fig. 263 in Pascher 1927) and a linear stigma. *Gloeomonas anomalipyrenoides* has a thinner mucus layer (c.a. 1 µm; Fig. 1H) and a circular to elliptical stigma. Although Ettl & Ettl (1959) depicted vegetative cells, they described *G. mucosa* as having an elliptic stigma. That form has a thick mucus layer and its flagellar bases are more distantly separated than in *G. anomalipyrenoides*. *Gloeomonas tubulosa* and *G. tecta* (Skuja) H. Ettl & O. Ettl also have a smooth chloroplast surface, but the flagellar bases of *G. tubulosa* are covered by a conspicuously long sheath. The vegetative cells of *G. tecta* are typically ellipsoid and not spherical (Ettl 1983). The other species of *Gloeomonas*, including *G. kupfferi* and *G. lateperforata*, have chloroplasts with a markedly reticular or perforated surface (Ettl 1983). *Chloromonas rubrifilum*, is also phylogenetically close to *Gloeomonas*, but it is also easily distinguished from *G. anomalipyrenoides*, because *C. rubrifilum* has ellipsoid vegetative cells (Ettl 1983, Pröschold *et al*. 2001).

Chloromonas vernalis (Skuja) Nakada **comb. nov.** ≡ *Chlamydomonas vernalis* Skuja in Nova Acta Regiae Soc. Sci. Upsaliensis. Ser. 4. 16(3): 138. 1956. *Chloromonas tapeta* var. *vernalis* H. Ettl in Nova Hedwigia Beih. 34: 130. 1970.

Typus. Skuja (1956), table 20: fig. 2 (**lectotype, designated here**; Sweden, Norrviken), specimen TNS-AL-58901 (**epitype, designated here**; Fig. 5) conserved at TNS; permanent preparations of vegetative cells from culture strain ACOI-3241.

Description. (Based on ACOI-3241) Mature vegetative cells nearly spherical; young cells sometimes ellipsoid (Fig. 2A, B), with thick bilayered cell wall; cell wall with low to high rounded, keel-like papilla (Fig. 2B, E–G). Papilla rounded-trapezoidal in front view (Fig. 2F), broadly triangular in side view (Fig. 2G). Cells without distinct mucus covering (Fig. 2E). Flagella two or three, emerging from edges of papilla, without sheaths (Fig. 2F, H, I). Flagella 1.0–1.5 × cell length. Basal bodies nearly in contact with each other (Fig. 2F). Chloroplast deep-cup shaped, with lobed inner surface (Fig. 2B); surface reticulately incised (Fig. 2A). No typical pyrenoid covered by starch granules, instead, small smooth patches corresponding to atypical pyrenoids on tip of chloroplast lobes (Fig. 2B, D). The stigma circular and positioned in the anterior region of the cell (Fig. 2A, C). Stigmata sometimes two. Nucleus in center of cell (Fig. 2B). Contractile vacuoles two or three, appearing close together in apical region (Fig. 2J, K). Cells 10–25 µm in diameter. Asexual reproduction accomplished by formation of two or four zoospores (Fig. 2L, M).

Phylogeny. Bayesian analysis (Fig. 3) shows *Chloromonas vernalis* ACOI-3241 to be sister to *C. kasaiae* Matsuzaki, Nakada, Y. Hara & Nozaki EbCl-8 (1.00 PP and 99–100% BPs). Although both ACOI-3241 and NIES-447 were previously identified as *C. insignis*, in the present study, they were clearly separated from each other. A previous three gene (18S *r*RNA, *atp*B, and *psa*B) phylogeny moderately supported sister relationship between *C. kasaiae* and *C. augustae* (Skuja) Pröschold, Marin, Schlösser & Melkonian SAG 5.73 (Matsuzaki *et al*. 2013; note that *C. vernalis* and the other strains of *C. augustae* were not included in the analyses).

Distribution. Sweden (Skuja 1956) and Portugal (ACOI-3241).

Strain. ACOI-3241 (labeled *Chloromonas insignis*).

Remarks. Among the species of *Chloromon-*

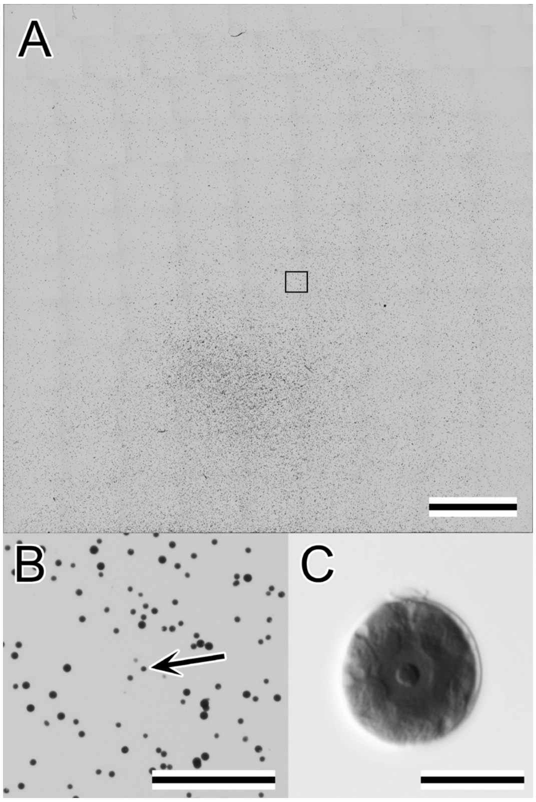

FIG. 5. Permanent preparation of *Chloromonas vernalis* (Skuja) Nakada comb. nov. (TNS-AL-58901; epitype). A: Overview of the preparation, shown as a composite micrograph. The region enclosed in a square is enlarged in (B). B: Vegetative cells in the epitype. Arrow indicates the cell shown in (C). C: Detail of a fixed vegetative cell in the epitype. Scale bars = 3 mm (A), 300 μm (B), or 10 μm (C).

as, nine usually have spherical cells greater than 10 μm in diameter, hemispherical, or truncated to keel-like papillae and a deep-cup shaped chloroplast with reticulate surface (Skuja 1956, Ettl 1983). ACOI-3241 was most similar to *Chloromonas vernalis* in morphological details of the papilla, cell covering, flagella, stigma and cell size (Table 1). Although ACOI-3241 was originally identified as *C. insignis*, it is distinguishable from *C. insignis* by a large papilla (Fig. 2B, F), absence of mucus (Fig. 2E) and a circular stigma (Fig. 2C). Strain ACOI-3241 could also be distinguished from its sister species, *C. kasaiae*, which has high, hemispherical papillae and usually ellipsoid cells (Matsuzaki *et al*. 2013).

The morphology of ACOI-3241 is essentially the same as in the original description of *Chlamydomonas vernalis* (Skuja 1956), but we observed several differences. The papillae of a strain from Portugal were narrower in side view than in front view (Fig. 2F, G), which we describe as rounded keel-like; Skuja (1956) described them as hemispherical without illustrating them in side view. The strain from Portugal also had a bilayered cell wall (Fig. 2E); Skuja (1956) described it as a thick cell wall. Bilayered cell walls have been depicted in *C. corticata* H. Ettl & O. Ettl and *C. ruzickae* Fott, but the cells of those species are clearly different from those of *C. vernalis*, as *C. corticata* has a two-humped papilla and a smooth chloroplast, and *C. ruzickae* has ellipsoid cells lacking a papilla. The outer layer of the cell wall of ACOI-3241 is sometimes thin and inconspicuous and might not have been well developed in the sample observed by Skuja (1956). Strain ACOI-3241 showed several aberrant morphological traits, such as three flagella (Fig. 2I), two stigmata, and three contractile vacuoles (Fig. 2K). Such cells are atypical and may possibly be developmental errors.

Ettl (1970) treated *Chloromonas vernalis* as a variety of *C. tapeta* (Skuja) Gerloff & H. Ettl without discussion. Although the diagnostic characters distinguishing *C. tapeta* and *C. vernalis* are quantitative, such as thickness of the cell wall and the size of the papilla and stigma (Table 1), combinations of these characters and the differences found in the original figures are significant (Skuja 1956) for distinguishing them. There is also no phylogenetic evidence to support a close relationship between these taxa. They are here treated as independent species, following Skuja (1956).

Conclusions

The delimitation of *Gloeomonas* from *Chloromonas* is problematic because species of *Gloeomonas* are phylogenetically nested within *Chloromonas* and there are several intermediate

TABLE 1. Differential characteristics of the genus *Chloromonas* with large spherical cells (≥ 12 µm), hemispherical papilla, and deep-cup shaped chloroplast with reticular surface (Skuja 1956, Ettl 1983). Taxa: 1. *C. tapeta*; 2. *C. vernalis* (≡*C. tapeta* var. *vernalis*); 3. *C. insignis*; 4. *C. pseudoplatyrhyncha*; 5. *C. porosa*; 6. *C. korschikoffii* (≡ *C. maculata*); 7. *C. alaskensis*; 8. *C. pseudopolychloris*; 9. *C. hovassei*. Abbreviations: +, present; −, absent; 1+, slightly longer; 1−, slightly shorter; hemisph., hemispherical; keel, keel-like; trunc., truncated.

	ACOI-3241	1	2*	3	4	5	6	7	8	9
Papilla										
Size	Large	Low	Large	Low	Large	Low	Low	Small	Low	Large
Shape	Keel	Hemisph.	Hemisph.	Hemisph.	Keel	Trunc.	Keel	Hemisph.	Hemisph.	Trunc.
Cell wall	Thick (bilayer)	Thin	Thick	Thick	Thick	Thick	Thin to thick	Thick	Thick	Thick
Mucus coverage	−	−	−	+	−	−	−	−	+	−
Flagella*	1–1.5	1+	1+	1	1+	1−	1	1	1.5	1
Stigma										
Shape	Circular	Circular to elliptical	Circular	Elliptical	Elliptical	Circular?	Elliptical to linear	Elliptical	Elliptical	Circular or longer
Position	Anterior	Middle	Middle to anterior	Anterior	Middle	Anterior	Anterior	Anterior	Anterior	Middle
Cell length (µm)	10–25	10–22	15–25	18–23	18–22	19–31	15–22	12–21	13.5–19	17–23

* Ratio of flagellar length to diameter of cell.

forms (Nozaki *et al*. 2010). Although close morphological examination of *Chloromonas rubrifilum* is required, available cultures of *Gloeomonas* including *G. anomalipyrenoides* are closely related to each other, and evolution of more or less distant flagellar bases is strongly indicated (Nozaki *et al*. 2010). The genus *Gloeomonas* in the traditional sense would therefore also be natural in a phylogenetic sense. Because *G. anomalipyrenoides* is one of the basal lineages of *Gloeomonas* (Nozaki *et al*. 2010) and *C. vernalis* might be related to *Gloeomonas* (Fig. 3), their recharacterization would improve the understanding of *Gloeomonas*. Close examination of related strains, such as *Gloeomonas* SAG 6.91 and *C. rubrifilum* SAG 3.85, is encouraged before redefining *Gloeomonas*. Although *C. vernalis* is more closely related to the species of *Gloeomonas* than to the type species of *Chloromonas*, *C. reticulata* (Fig. 3), it has close flagellar bases and therefore cannot be assigned to *Gloeomonas*. For a phylogenetically natural classification, the paraphyletic genus *Chloromonas* should be divided into monophyletic genera. Morphological characterization of the major clades within *Chloromonadinia* is necessary. Such a revision might transfer *C. vernalis* from *Chloromonas* to a different (possibly new) genus.

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